Phytogeographic Analyses of Variation in *Cimicifuga simplex* (Ranunculaceae) Based on Internal Transcribed Spacer (ITS) Sequences of Nuclear Ribosomal DNA

Hiroki YAMAJI, Iwao SAKAKIBARA, Kenji KONDO, Mao SHIBA,
Eiji MIKI, Nobuyuki INAGAKI, Susumu TERABAYASHI,
Shuichi TAKEDA and Masaki ABURADA*

Pharmacognosy & Medicinal Resources Laboratory, Research Division, Tsumura & Co.,
3586, Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki, 300-1192 JAPAN
E-mail: yamaji_hiroki@mail.tsumura.co.jp
*Present address: Department of Pharmaceutical Sciences, Faculty of Pharmacy, Musashino University,
1–1–20 Shin-machi, Nishitokyo-shi, Tokyo, 202-0023 JAPAN

ITS variation in *Cimicifuga simplex* (Ranunculaceae) was studied in 53 populations from Japan. Thirty-one sites were observed as polymorphic among the *C. simplex* populations. Seven genotypes composed of four ribotypes (Types 1–4) were recognized. Four homozygote types (1–4) and three heterozygote types (Types 1 + 2, 1 + 3 and 3 + 4) were included in the seven genotypes.

Distinct patterns were observed in the distributional range of the four ribotypes. Ribotype 1 was distributed in central Honshu. The range of ribotype 2 covered the region from the Pacific Ocean side of northern Honshu to central Honshu, and disjunctively extended to the region at an elevation of 1600 m or more in Shikoku and Kyushu, western Japan. Ribotype 3 was most widely distributed throughout Japan. Ribotype 4 occurred in the region at an elevation of 1000 m or less in Shikoku and Kyushu. Differences in the range of the four ribotypes (especially homozygotes) corresponded to those of environmental conditions in the habitats. Homozygotes within ribotype 2 inhabited the sites of higher altitude similar to boreal plants. Homozygotes of both ribotypes 1 and 4 were restricted to the region of comparatively mild climate such as sites of lower altitude or western longitude (it becomes warmer towards west in Japan). It is accordingly concluded that the *Cimicifuga simplex* includes phylogenetically distinct races adapted to different environmental conditions.

Key words: *Cimicifuga simplex*, environmental preferences, heterozygotes, ITS, phylogeography.

Intraspecific phylogeography based on molecular information provides new insights into species history (Avise 2000). For Japanese plants, only several studies have been reported so far; *Fagus crenata* (Tomaru et al. 1998, Fujii et al. 2002), *Abies* spp. (Tsumura and Suyama 1998), *Stachyurus praecox* (Ohi et al. 2003a), *Aucuba* spp. (Ohi et al. 2003b), and a few Japanese alpine plants (Fujii et al. 1997, 1999).

Internal transcribed spacer (ITS) of nuclear ribosomal DNA is the most exploited source of molecular data for systematic studies of plants, especially for intrageneric and intraspecific variation (Hershkovitz et al. 1999). In contrast to genetic markers in

cpDNA and mtDNA, which are frequently used in phylogeographic studies (Hillis and Moritz 1990, McCauley 1994, Fujii et al. 2002), ITS is biparentally inherited, influenced by pollen flow, tandem multicopied, and sometimes recombines (Baldwin et al. 1995, Hershkovitz et al. 1999).

ITS retains traces of hybridization in the past as heterozygotes of phylogenetically different ribotypes in their tandem multicopied array (Baldwin et al. 1995, Sang et al. 1995), and may enlarge gene flow effects, eliminate misunderstanding caused by chloroplast capture. Therefore, ITS variation may have advantage than cpDNA variation to recognize geographic structures formed by seed and pollen flows including hybridization between isolated populations.

We examined a perennial herb, *Cimicifuga simplex* (DC.) Wormsk. ex Turcz. (Ranunculaceae), distributed in East and Northeast Asia: Japan, Korea, northeast and south China, Sakhalin, the Kurile Islands, Kamchatka, and east Siberia (Hsiao 1979, Tamura 1982, Compton et al. 1998b). This species is suitable for a phylogeographic study for Japanese plants because it is widely distributed and comparatively common. Moreover, Compton et al. (1998a) reported that intraspecific variation of this species in 10 sites of ITS sequences based on four geographically different materials.

In this study, we examined the phylogeographic structure of *C. simplex* from throughout Japan based on ITS sequences from 58 populations. At the same time, we compared the variation of ITS sequences in this study with those recognized by Compton et al. (1998a), and inferred whether *C. simplex* is divided into more than one genetically distinct type.

Materials and Methods
Field collections
Field collections were made from 1999 to 2000. Fifty-three populations of *Cimicifuga*

simplex were examined (Table 1). To understand the overall pattern of geographic variation in ITS within *C. simplex*, one individual from each population was examined. Four sequences belonging to *C. simplex* reported by Compton et al. (1998a) were obtained from DDBJ/EMBL/GenBank database. For outgroups in phylogenetic analysis, six materials belonging to three putative sister group species as inferred by Compton et al. (1998 a, b), *C. dahurica* (Turcz.) Maxim., *C. foetida* L., *C. heracleifolia* Kom., and one outgroup species: *C. biternata* (Siebold & Zucc.) Miq., were also examined (Table 1). The voucher specimens were deposited in THS.

Genetic analysis
Total DNA was extracted using the DNA-easy® Plant Mini Kit (QIAGEN) from fresh leaf or dried materials for each material. To detect the complete sequence of the ITS region, the universal primer set of White et al. (1990), ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) was used for amplification by PCR. The PCR reaction mixture consisted of 10 × Gene Taq Buffer (Nippon Gene) 5 µl, dNTP mix (Nippon Gene) 4 µl, forward primer (ITS5: 10 pmol/ml) 1 µl, reverse primer (ITS4: 10 pmol/ml) 1 µl, Gene Taq (Nippon Gene) 0.25 µl, DMSO 5µl, D.D.W. 32.5 µl, and template DNA 1.25 µl. PCR cycling condition was as follows: (94°C, 4 min) × 1 cycle, (94°C, 1 min; 48°C, 2 min; 72°C, 3 min) × 30 cycles, and (72°C, 7 min) × 1 cycle. The PCR products were purified by electrophoresis in 1.0 % TAE agarose gel stained with ethidium bromide, and GFX™ PCR DNA and Gel Band Purification Kit (Amersham biotech). We sequenced the purified PCR products using the BigDye Terminator Cycle Sequencing Kit ver. 2.0 and Model 3100 automated sequencer (Applied Biosystems), following the manufacturer's instructions.

Table 1. Species name, collection site, altitude, voucher specimen of *Cimicifuga* materials examined

No.	Locarity	voucher
Cimicifuga simplex		
Hokkaido		
1	Abashiri, Rubeshibe, alt. 200 m	THS 34186
2	Tokachi, Kamishihoro, alt. 400 m	THS 35078
3	Hidaka, Urakawa, alt. 100 m	THS 41827
4	Shiribeshi, Shimamaki, alt. 50 m	THS 41828
5	Oshima, Shiriuchi, alt. 100 m	THS 41825
Tohoku		
6	Aomori, Kawauchi, alt. 50 m	THS 41823
7	Akita, Akita, alt. 250 m	THS 42319
8	Akita, Tazawako, alt. 300 m	THS 67085*
9	Akita, Kosaka, alt. 350 m	THS 42322
10	Iwate, Tamayama, alt. 465 m	THS 42325
11	Miyagi, Naruko, alt. 250 m	THS 42314
12	Yamagata, Atsumi, alt. 150 m	THS 67017*
13	Yamagata, Nishikawa, alt. 500 m	THS 60660*
14	Yamagata, Kaminoyama, alt. 600 m	THS 60681*
15	Fukushima, Inawashiro, alt. 570 m	THS 42328
Kanto		
16	Gunma, Tsukiyono, alt. 860 m	THS 41761
17	Tochigi, Nikko, alt. 800 m	THS 42332
18	Ibaraki, Kitaibaraki, alt. 100 m	THS 41563
19	Ibaraki, Takahagi, alt. 100 m	THS 41847
20	Ibaraki, Tsukuba, alt. 400 m	THS 42336
21	Tokyo, Okutama, alt. 400 m	THS 42472
22	Kanagawa, Minamiashigara, alt. 800 m	THS 66918*
23	Kanagawa, Hadano, alt. 1100 m	THS 67086*
24	Chiba, Amatsukominato, alt. 270 m	THS 42566
Chubu		
25	Niigata, Yunotani, alt. 1320 m	THS 42334
26	Niigata, Maki, alt. 80 m	THS 67084*
27	Yamanashi, Kawaguchiko, alt. 1600 m	THS 42091
28	Shizuoka, Oohito, alt. 280 m	THS 41018
29	Shizuoka, Matsuzaki-cho, Akebushi, alt. 100 m	THS 42570
30	Shizuoka, Matsuzaki-cho, Fukino, alt. 540 m	THS 42575
31	Nagano, Matsukawa, alt. 700 m	THS 42569
32	Nagano, Chino, alt. 1160 m	THS 42568
33	Nagano, Karuizawa, alt. 1400 m	THS 67083*
34	Nagano, Kinasa, alt. 650 m	THS 41822
35	Gifu, Osaka, alt. 665 m	THS 42522
36	Gifu, Kiyomi, alt. 830 m	THS 42521
37	Gifu, Shirotori, alt. 680 m	THS 42519
38	Toyama, Toga, alt. 500 m	THS 41821
39	Ishikawa, Kanazawa, alt. 200 m	THS 67075*
40	Ishikawa, Tsurugi, alt. 400 m	THS 67076*
41	Fukui, Ohama, alt. 130 m	THS 42550
Kinki		
42	Shiga, Maibara, alt. 460 m	THS 42548
43	Osaka, Chihayaakasaka, alt. 870 m	THS 42564
44	Hyogo, Shingu, alt. 500 m	THS 67060*
45	Hyogo, Onsen, alt. 415 m	THS 42522
Chugoku		
46	Shimane, Yokota, alt. 675 m	THS 42554
47	Yamaguchi, Yamaguchi, alt. 220 m	THS 42559
48	Hiroshima, Togouchi, alt. 640 m	THS 42556
Shikoku		
49	Tokushima, Koyadaira, alt. 1760 m	THS 42544
50	Ehime, Kawauchi, alt. 950 m	THS 42541
Kyushu		
51	Fukuoka, Soeda, alt. 600 m	THS 42562
52	Fukuoka, Maebaru, alt. 525 m	THS 42561
53	Miyazaki, Shiiba, alt. 1600 m	THS 42563
Sequences examined by Compton et al. (1998a)		
54	Z98298 Taiwan***	
55	Z98299 Honshu***	
56	Z98300 Hokkaido***	
57	Z98301 S. Korea***	
Outgroups		
C. dahurica		
58	China, Liaoning	THS 00671**
59	China, Jilin	THS 00856**
C. heracleifolia		
60	China, Liaoning	THS 00557–2**
C. foetida		
61	China, Yunnan	THS 00562**
62	China, Sichuan	THS 00506–1**
C. biternata		
63	Ibaraki, Tsukuba	THS 00678**

*medicinal part specimen. **cultivated strain. ***cited from DDBJ/EMBL/GenBank database.

For sequencing, we used the same primers as those used for amplification. Sequences were aligned manually. All variant characters were compared in raw data of the automated sequencer, and ambiguous (additive) sites were coded according to the IUPAC (IUB) codes.

The phylogenetic relationships of the materials were inferred by the maximum-persimony (MP) method. For the analysis, we used PAUP* 3.1.1 program (Swofford 1993). All characters were weighted equally, and all indels were coded as present/absent data. The MP analysis was conducted through a heuristic search with TBR branch-swapping option. Bootstrap analysis (Felsenstein 1985) with 1000 replications was performed with the same program.

Results
Variation in ITS sequences of *Cimicifuga simplex*

Complete nucleotide sequences of ITS1, 5.8S rDNA, and ITS2 are deposited in the DDBJ/EMBL/GenBank database under the accession numbers listed in Fig. 1. The lengths of all materials of *C. simplex* examined in this study were 225 bp (ITS1), 167 bp (5.8S rDNA), and 212 bp (ITS2) long. Although each of the four ITS sequences belonging to *C. simplex* reported by Compton et al. (1998a) had one or two-bp deletions, there were no distinct indels among the ITS sequences of materials belonging to *C. simplex* examined in this study.

The five species examined in this study were discriminated from each other by their specific diagnostic sites: *C. dahurica* (No. 58: AB194178, No. 59: AB194179), *C. heracleifolia* (No.60: AB194180), *C. foetida*, (No. 61: AB194181, No. 62: AB194182), and *C. biternata* (No. 63: AB194183).

Within the 53 materials in this study belonging to *C. simplex*, 31 variable sites were recognized (Fig. 1). On the other hand, the number of variable sites within *C. simplex*

reported by Compton et al. (1998a) was 12. Only five variable sites were shared between them. The total number of variable sites was 38. These variable sites were numbered 1–38 (Fig. 1).

The 38 variable sites were classified into four types by number and condition of observed states of nucleotide (Fig. 1). The first was that two states e. g. 'G' or 'C' were recognized, and no additive states were recognized (sites 1–7). This type was recognized only in the sequences reported by Compton et al. (1998a). The second was that three states of nucleotides e. g., 'G', 'A' and their additive state 'R' ('G' + 'A') were recognized. This type was recognized in four sites (sites 8–11). The third was that two states, e. g., 'C' and additive state 'Y' ('C' + 'T') were recognized in more than one material, respectively. This type was recognized in 13 sites (sites 12–24). The fourth was that two states (e. g., 'C' and 'Y') were recognized, and the additive states were recognized in only one material. This type was recognized in 14 sites (sites 25–38).

Our data set included more sites in additive states than that of Compton et al. (1998a), probably because the criteria to recognize additive states in each site differs among researchers. For example, at site 11, the state of material No. 60 reported by Compton et al. (1998a) was 'C', however it might be possible that it was 'T' or 'Y' in our interpretation, because we found no individuals with state 'C' in this site. Then we compared the variable sites recognized in this study only in the following analyses, and treated site 11 as the third type variation.

In combination of the states of the second type variations (sites 8–10), we recognized seven ITS genotypes (Fig. 1). Among the seven ITS genotypes, four types were without any additivities e. g. 'Y' or 'R' in these sites, we call them homozygote types, composed of only one of ribotype 1–4, respectively (we call them homozygote type 1–4).

A total of 43 (70 %) materials came under them, 6 (10 %), 10 (16 %), 22 (36 %), and 5 (8 %) materials came under homozygote type 1, 2, 3, and 4, respectively. The other three types were with one additive site in these sites. They were able to be explained as heterozygotes between two of ribotypes 1–4: 1 + 2, 1 + 3, and 3 + 4 (we call them heterozygote types 1 + 2, 1 + 3, and 3 + 4, respectively). A total of 18 (30 %) materials came under such heterozygote types, 4 (7 %), 8 (13 %), and 6 (10 %) came under heterozygote type 1 + 2, 1 + 3, and 3 + 4, respectively (Fig. 1).

Among the sequences reported by Compton et al. (1998a), those from Taiwan (No. 58: Z98298) and Honshu (No. 59: Z98299) came under homozygote type 1, and in the same way, that from S. Korea (No. 61: Z98301) was of homozygote type 2, and that from Hokkaido (No. 60: Z98300) was from homozygote type 3.

The third type variations could almost all be explained as variations accompanied with the four ribotypes recognized by the second type variation with a few exceptions. For example, materials with additive states in site 18 were those with ribotype 3 (homozygote type 3 or heterozygote type 3 + 4), and individuals with additive states in site 21–24 came under with ribotype 4 (homozygote type 4 and heterozygote type 3 + 4). The fourth type variations were regarded as autapomorphies, and had little information for phylogenetic reconstruction and phylogeographic grouping.

Therefore, we discuss mainly the seven genotypes recognized by the second type variable sites (sites 8–10) hereafter, because they are appropriate to estimate nucleotide substitutional pattern phylogenetically.

Phylogenetic reconstruction of *C. simplex*

We conducted a molecular phylogenic analysis in the following conditions. First, we excluded the first, third, and fourth type variable sites (sites 1–7, 11–38) from the ITS sequences of each materials. The reasons are as follows. The first type variable sites (sites 1–7) were not recognized in this study. At the third and the fourth type variable sites (sites 11–38), it is difficult to draw a line between additive and non additive condition in some materials, and it would confuse phylogenetic trees. Second, individuals that came under the heterozygote types in the second variable sites (sites 8–10) were excluded, because they were supposed to originate in hybridization between the four homozygote types, and confuse phylogenetic reconstruction. Third, materials which came under the same homozygote type were made up to single OTUs, respectively (ribotypes 1–4), because they are the same except sites 1–7, 11–38, which are excluded in the first condition.

Only one maximally parsimonious tree (MPT) was obtained (Fig. 2). *Cimicifuga simplex* was monophyletic which is supported by three unique apomorphic substitutional sites. The most closely related species toward *C. simplex* were *C. dahurica* and *C. heracleifolia* in the MPT. Within *C. simplex*, four ribotypes split into one basal grade and two major clades; ribotype 1 was in the basal grade, clade 1 was composed of ribotype 2, and clade 2 was composed of ribotype 3 and 4. Clade 2 was further divided into one monophyletic clade (ribotype 4) and a basal grade (ribotype 3).

Distinct patterns were observed among the distributional ranges of the four ribotypes composing the seven ITS genotypes (Fig. 3). Ribotype 1 was distributed in central Honshu. Homozygote type 1 was restricted mainly to the southern part of central Honshu (Izu and Boso Peninsulas). Ribotype 2 was distributed in the region from the Pacific side of northern Honshu to central Honshu, and disjunctively extended to the areas at an elevation of 1600 m or more in Shikoku and Kyushu, western Japan. Homo-

zygote type 2 was distributed in almost all areas where ribotype 2 existed. Ribotype 3 was the one most widely distributed throughout Japan. Homozygote type 3 was observed mainly in Hokkaido, northern Honshu and central Honshu (on the Sea of Japan side). Ribotype 4 was distributed in the region at an elevation of 1000 m or less in western Honshu, Shikoku and Kyushu. Homozygote type 4 was distributed in almost all areas

site number: 1 2 3 4 5 6 7 | 8 9 10 | 11* 12 13 14 15 16 17 18 19 20 21 22 23 24 | 25 26 27 28 29 30 31 32 33 34 35 36 37 38

position: 18 26 213 494 543 591 603 | 50 418 565 | 558 191 59 117 33 44 68 468 199 422 467 484 208 | 49 103 111 212 217 306 421 476 547 552 559 561 566 576

material No.	type 1	type 2	genotype	type 3	type 4	accession No.
32	GCGCCAC	GGC	①	TCCCACCTAGGCCG	TCGCCGGGGGACTC	AB194140
30	GCGCCAC	GGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	
24	GCGCCAC	GGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	AB194141
29	GCGCCAC	GGC		TCYCACCTAGGCCG	TCGCCGGGGGACTC	
59	C-GCGAC	GGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	·
58	G-GTCTC	GGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	·
34	GCGCCAC	RGC	①+②	TYCCACCTAGGCCG	TYGCCGGGGGACTC	AB194142
31	GCGCCAC	RGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	AB194143
15	GCGCCAC	RGC		TCCYACCTAGGCCG	TCGCCGGGGGACTC	AB194144
36	GCGCCAC	RGC		TCCYACCTAGGCCG	TCGCCGGGGGACTC	
53	GCGCCAC	AGC	②	TCCYACYTAGGCCG	TCGCCGGGKGACTC	AB194145
11	GCGCCAC	AGC		TCCYACCTAGGCCG	TCGCYGGGGGACTY	AB194146
49	GCGCCAC	AGC		TCCYACCTAGGCCG	TCGCCGGGGGACTC	AB194147
17	GCGCCAC	AGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	AB194148
33	GCGCCAC	AGC		TCCCACCTAGGCCG	TCGCCGGGGGACTC	
10	GCGCCAC	AGC		TCCCACCTAGGCCG	TCGCCGGGGGRCTC	AB194149
61	G-GCCA-	AGC		TCCCACCTAGGCCG	TCGYCGGGGGACTC	·
21	GCGCCAC	GKC	①+③	TYYCACCTAGGCCG	TCGCCGGGGGACTC	AB194150
22	GCGCCAC	GKC		TCYCRCCTAGGCCG	TCGCCGGGGGACTC	
35	GCGCCAC	GKC		TCYCRCCTAGGCCG	TCGCCGGGGGACTC	AB194151
23	GCGCCAC	GKC		TCYCAYCTAGGCCG	TCGCCGGGGGACTC	AB194152
19	GCGCCAC	GKC		TCYCAYCTAGGCCG	TCGCCGGGGGACTC	
20	GCGCCAC	GKC		TCYCACCTAGGCCG	TCGCCGGGGGACTC	AB194153
18	GCGCCAC	GKC		TCYCACCTAGGCCG	TCGCCGGGGGACTC	
28	GCGCCAC	GKC		TCYCACCTAGGCCG	TCGCCGGGGGACTC	
27	GCGCCAC	GTC	③	TCCCACCTAGGCCG	YCGCCGGGGGACTC	AB194154
37	GCGCCAC	GTC		TCYCACCTAGGCCG	TCGCCGGGGGACTC	AB194155
14	GCGCCAC	GTC		TCCCACCTAGGCCG	TCGCCRGGGGACTC	AB194156
26	GCGCCAC	GTC		YCCCACCARGCCG	TCGCCGRGGGACTC	AB194157
38	GCGCCAC	GTC		YCCCACCARGCCG	TCGCCGGGGGACTC	AB194158
6	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	
13	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	
4	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	
16	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	AB194159
5	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	
9	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	
7	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGGACTC	
8	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGRGGACTC	AB194160
25	GCGCCAC	GTC		YCCCACCYAGGCCG	TCSCCGGGGGACTC	AB194161
12	GCGCCAC	GTC		YCCCACCYAGGCCG	TCGCCGGGGAYYC	AB194162
2	GCGCCAC	GTC		YCCCACYAGGCCG	TCGCCGGGGGACTC	AB194163
1	GCGCCAC	GTC		YCCCACYTAGGCCG	TCGCCGGGGGACTC	AB194164
3	GCGCCAC	GTC		YCCCACYTYGGCCG	TCGCCGGGGGACTC	AB194165
40	GCGCCAC	GTC		YCCCACCTMGGCCG	TCGCCGGGGGACTC	AB194166
39	GCGCCAC	GTC		YCCCACCTAGGCCG	TCGCCGGGGGACTC	AB194167
60	G-ACCT-	GTC		CCCCACCTAGGCCG	TCGCCGGGGGACTC	·
41	GCGCCAC	GTY	③+④	TCYCACCTAGGCCG	TCGCCGGGGGACTC	AB194168
50	GCGCCAC	GTY		TCYCACCTAGGCCG	TCGCCGGGGGACTC	
44	GCGCCAC	GTY		TCYCACCTAGKCYG	TCGCCGGGGGACTC	AB194169
51	GCGCCAC	GTY		TCYCACCTAGRCCG	TCGCCGGGGGACTC	AB194170
47	GCGCCAC	GTY		TCYCACCTAGRYCG	TCGCCGGGGGACTC	AB194171
42	GCGCCAC	GTY		TCYCACCTAGRYK	TCGCCGGGGGACTC	AB194172
43	GCGCCAC	GTT	④	TCYCACCTAGRCYK	TCGCCGGGGGACTC	AB194173
48	GCGCCAC	GTT		TCYCACCTAGGYCG	TCGCCGGGGGACTC	AB194174
52	GCGCCAC	GTT		TCYCACCTAGRYCG	TCGCCGGGGGACTC	AB194175
45	GCGCCAC	GTT		TCYCACCTAGRCCG	TCGCCGGGGGACTC	AB194176
46	GCGCCAC	GTT		TCYCACCTAGGCCG	TCGCCGGGGGACTC	AB194177

material number****: type 1 — 1(1) 4(4) 1(1) 1(1) 2(2) 2(2); type 2 — 11(7) 40(32) 11(5); type 3 — 19(1) 23 5 2 2 4 14 2 2 7 4 3 2; type 4 — 1 1 1 1 1 1 1 1 1 1 1 1 1 1
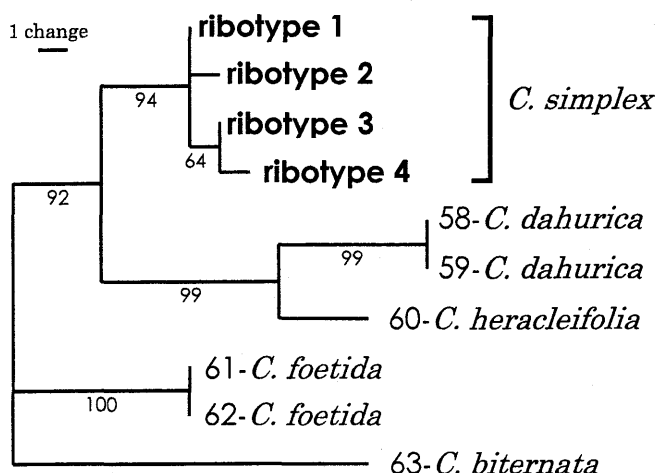
Fig. 2. Only one maximally parsimonious tree (MPT) of *Cimicifuga simplex* and its allied species with the ITS sequences. The MPT have 41 steps, a CI of 0.951 (0.929 excluding uninformative characters), and a RI of 0.951. Numbers below branches are bootstrap values in 1000 replicates.

where ribotype 4 was found. Heterozygote types 1 + 2 and 1 + 3 were recognized in central Honshu, where is a border between the distribution ranges of homozygote plants of ribotypes 1, 2, and 3. Heterozygote type 3 + 4 was recognized in western Honshu, where homozygotes of ribotype 4 were observed.

Discussion

For *Cimicifuga simplex* in Japan, the ITS variation recognized in this study was different from that presented by Compton et al. (1998a). Our data set included more sites in additive states than those of the previous study. Moreover, we could not recognize the seven variable sites reported by the previous study, and they are doubtful at least in the sequences from populations in Japan (Fig. 1: Nos. 55, 56). Compton et al. (1998b) divided *C. simplex* into four distinct species under the genus *Actaea*: *A. simplex* (DC.) Wormsk. ex Prantl, *A. taiwanensis* J. Compton, Hedd. & T. Y. Yang, *A. yesoensis* (Nakai) J. Compton & Hedd., and *A. matsumurae* (Nakai) J. Compton & Hedd. These species

Fig. 1. Character states of each materials in variable sites within *Cimicifuga simplex* in ITS sequences. Sequences obtained by Compton et al. (1998a) were reversed in black and white. *Classified into the third type variation because the only materials with 'C' was those obtained by Compton et al. (1998a), and it is doubtful. **Type 1: variable sites that two types were recognized without intermediate status, e. g., 'G' or 'C', type 2: three conditions were recognized, e. g., 'G', 'A', and their additive status 'R', type 3: two conditions were recognized, e. g., 'C' and 'Y' (intermediate status between 'C' and 'T'), and the intermediate states were shared in more than one samples, type 4: two conditions were recognized, e. g., 'C' and 'Y', and the intermediate states were peculiar to only one sample, respectively. ***Seven genotypes recognized in the site 8-10, expressed as the combination of the four ribotype (①–④). ****Number of materials with site substitution within *C. simplex*. That with complete site substitution are shown in parenthesis.

are recognized based on morphological characters, the ITS sequences (Compton et al. 1998a) and the sequences of *trnL* intron, *trnL-F* intergenic of chloroplast DNA (Compton et al. 1998b). However, our preliminary morphological observation, it was difficult to classify plants belonging to *C. simplex* into the four species by the key presented by Compton et al. (1998b). For example, though Compton et al. (1998b) proposed that the delimitation between *A. simplex* and *A. matsumurae* by the proportion of central lobe of terminal leaflets to total length of leaflet and by the presence of hairs on the adaxial side of leaflets, these morphological attributes seemed to be continuous. In addition, the genetic supports were uncertain because only one entry for each species was examined (*A. simplex*: No. 59, Z98299, *A. taiwanensis*: No. 58, Z98298, *A. yesoensis*: No. 60, Z98300 and *A. matsumurae*: No. 61, Z98301). Comparing the seven ITS genotypes recognized in this study and the four sequences belonging to *C. simplex* presented by Compton et al. (1998a) in the sites 8–10, the homozygote types 1, 2, and 3 correspond to *A. matsumurae*/*A. taiwanensis*, *A. simplex* and *A. yesoensis*, respectively. However the ITS variation recognized in this study is negative to recognize more than one distinct types in *C. simplex* in Japan, because not a little heterozygote individuals between the homozygote types were recognized.

ITS sequences considered to be neutral and the distribution pattern of genotype probably reflect the historical process of speciation (Baldwin et al. 1995, Yokoyama et al. 2003). Hence, its variation probably reflects the genetic background derived from historical process of this species. For plants of the temperate zone such as *C. simplex*, their distributional ranges are supposed to be influenced by repeated contraction and expansion of their distribution during the glacial cycles (Hewitt 1996, 2000).

Our result indicates that the distribution

ranges of the four ribotypes, especially homozygote individuals corresponded to the difference of environmental condition. For example, the homozygote types 1 and 4 mainly inhabited in areas of mild climate (southern maritime regions of central Japan and westrn Japan). On the other hand, the homozygote types 2 and 3 were distributed mainly in regions of high altitude or high latitude like boreal plants in Japan: they occur in the region from northern Honshu to central Honshu (on the Sea of Japan side), and disjunctively in the regions at an elevation of 1600 m or more in Shikoku and Kyushu.

In order to explain the geographic pattern recognized in the ITS sequences, the following two hypotheses are proposed; first the four genetically different races segregated into different habitats , second the arrangement of the four races reflects routes of migration in the past. The former presumes that phylogenetically different races are also different in environmental preference. Both hypotheses agree with the comparatively wide range of distribution in *C. simplex* (from Kamchatka to southern China; Tamura 1982, Compton et al. 1998b).

The former hypothesis is rather suitable to explain the segregation of plants of ribotypes 2–4 by segregation in Shikoku and Kyushu because the beeline distance between them is comparatively short in comparison with their ranges. On the other hand, the latter hypothesis is suitable to elucidate the different ranges of ribotypes 1 and 4, which are supposed to be in similarly mild environmental conditions. The current phylogeographic pattern of *C. simplex* is therefore supposed to be formed by both factors.

On the basis of these observations, we estimated the process of the formation of phylogeographic structure of *C. simplex* in Japan as follows: ribotype 1 is phylogenetically most ancestral. The range of the homozygote type 1 is mainly limited to the

southernmost part of central Honshu. This distribution pattern corresponds to those of the "Fuji-Hakone element" proposed by Kanai (1958), which origin is considered to be old and well differentiated by long-term isolation. This area also supposed to have been one of the refugia for temperate plants in the glacial periods (Fujii et al. 2002, Ohi et al. 2003). Therefore it is possible that the plesiomorphic state of environmental preference of *C. simplex* is that of mild climate.

Ribotypes 2 and 3 are probably derived independently from ribotype 1. Populations with these ribotypes appears to have adapted to comparatively severe environmental conditions. It is noteworthy that the homozygote type 2 plants are distributed in areas at high altitude in Shikoku and Kyushu as relicts of boreal plants (Shimizu 1982, 1983). It should be noted that the ranges of ribotypes 2 and 3 were segregated into the Pacific Ocean side and the Japan Sea side of Japan (Fig. 3). Many phytosociological studies on Fagus crenata forests have been conducted in Japan since the 1950's, most of which revealed the presence two major alliances (or associations), one on the Pacific Ocean side and another on the Japan Sea side of the Japan

archipelago (Hukushima et al. 1995). A recent molecular phylogeographic study of *Fagus crenata* supported these two major alliances (Fujii et al. 2002). Phylogeographic pattern of *C. simplex* is similar to these cases.

Ribotype 4 is supposed to be derived from ribotype 3 in western Japan. It is possible that populations with ribotype 4 have adapted to comparatively mild environmental conditions.

To explain the situation that *C. simplex* includes three heterozygote types in addition to the four distinct homozygote types in ITS regions, we inferred the following process. Populations of *C. simplex* had been restricted to some segregated areas, and the four homozygote types were formed through the fixation of the variation of substitutional sites (sites 8–10). Thereafter, through local populations with different ribotypes expanded, they have contacted secondarily, and formed the three heterozygote types (1 + 2, 1 + 3, 3 + 4). It is noteworthy that neither heterozygote type 2 + 3 nor 2 + 4 were recognized, though their distributional ranges come in contact with each other or overlapped together in central Honshu, Shikoku
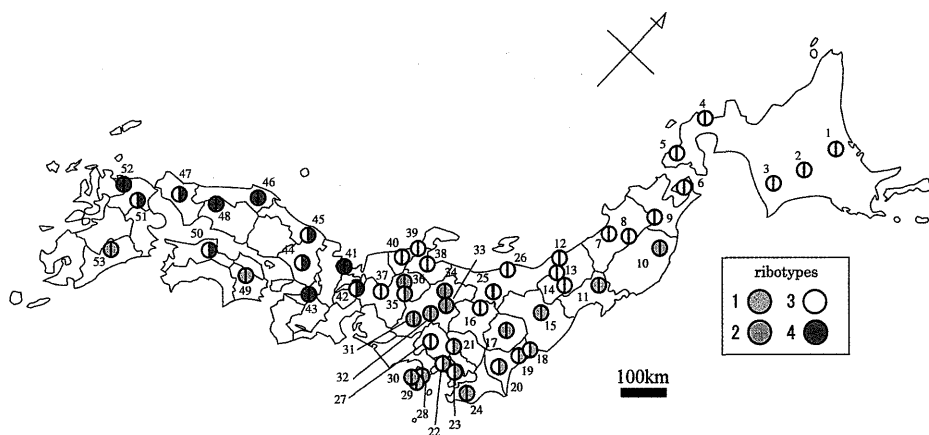

Fig. 3. Distribution of the four ribotypes recognized here in *Cimicifuga simplex*. Letters indicate sample number. Heterozygote types were indicated by color partition of circles.

and Kyushu. It is possible that a certain barrier (e. g., ecological isolation) may be present between plants with ribotypes 2 and 3 or 4.

Phylogeographic studies based on intraspecific variation of plants in Japan could be divided into two categories, one is for alpine or boreal plants (e. g., *Primula cuneifolia*: Fujii et al. 1997, 1999), another is for temperate plants in areas at comparatively lower altitude (e. g., *Fagus crenata*: Tomaru et al. 1998, Fujii et al. 2002, *Stachyurus praecox*: Ohi et al. 2003a, *Aucuba* spp.: Ohi et al. 2003b).

Disjunctive distribution in the alpine zone or northern regions is characteristic of the first hypothesis; populations may be in areas at higher altitude when they grow in the southern part of Japan, and populations located in the south are presumed to be isolated longer than the northern ones because they were genetically diversified from the northern ones (Fujii et al. 1999). Common occurrence in areas at lower altitude in the temperate zone is characteristic of the latter; a single or a few haplotypes of cpDNA were observed in northern area, on the contrary, many haplotypes were recognized in southern area (Ohi et al. 2003).

In the glacial periods, boreal plants are expected to expand their ranges to the south, and temperate plants are expected to contract their ranges to the south. In the interglacial periods, movements are expected in opposite directions to those in the glacial periods, respectively. These precedent studies do not present a hypothesis that a single species includes more than one phylogenetically differing race corresponding to difference of environmental preferences.

The present study suggests that the phylogeographic structure of *C. simplex* recognized here implies that a single species includes phylogenetically divergent races segregated into different environmental conditions. Although these races are expected to hybridize with each other in the present time, it is possible that such segregation promotes their speciation when their ranges constract due to certain environmental changes.

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山路弘樹，榊原　巌，近藤健児，司馬真央，三木栄二，稲垣伸行，寺林　進，竹田秀一，油田正樹*：核リボゾーム DNA，ITS 領域に基づく，キンポウゲ科サラシナショウマ属サラシナショウマの生物地理学的研究

　サラシナショウマ（キンポウゲ科）について，日本全国からの53集団を用いて ITS 領域の塩基配列における地理的変異を調査した．その結果，本種内には31サイトで多型が見いだされ，このうちの３サイトにおける変異パターンから，次のような７つのタイプが認められた．すなわち，これら

の３サイト内に個体内多型の見いだされない４タイプ（同型接合型タイプ１～４）と，個体内多型の見られる３タイプの，合計７タイプである．後者の３タイプは，前者のタイプのリボタイプ（リボタイプ１～４）を異なる組み合わせでもつタイプ（異型接合型タイプ１＋2，1＋3 および 3＋4）である．

　これらの７タイプを構成するリボタイプ１～4は異なる分布域をもつことが明らかになった．す

なわち，リボタイプ1は本州中部に，リボタイプ2は本州北部（太平洋側地域）〜中部，さらに四国と九州の高地に隔離的に分布していた．リボタイプ3は日本全国に広く分布し，リボタイプ4は本州西部，四国，九州の低標高地に分布していた．同型接合型タイプ2は西日本の高地に隔離的に分布しており，これは周北極植物の分布パターンに類似している．逆に，同型接合型タイプ1と4は比較的温暖な地域か西日本の低地に限られていた．以上のように，サラシナショウマの中には，異なる環境条件に適応したそれぞれ異なる系統が含まれていることが明らかになった．

（（株）ツムラ研究本部生薬・資源研究所，
*現所属：武蔵野大学薬学部）